APLICACIONES DEL CELL SORTING EN ENFERMEDADES ONCOHEMATOLOGICAS

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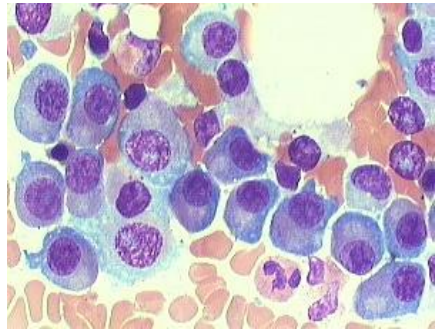
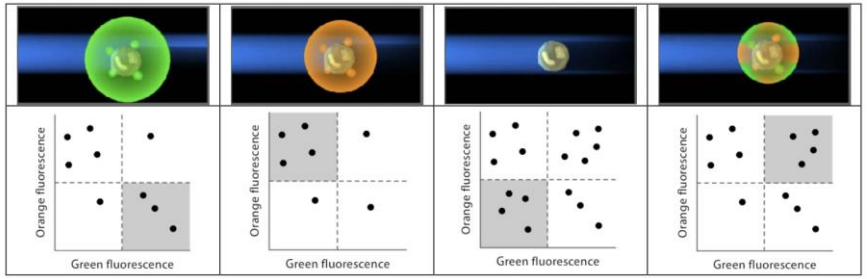
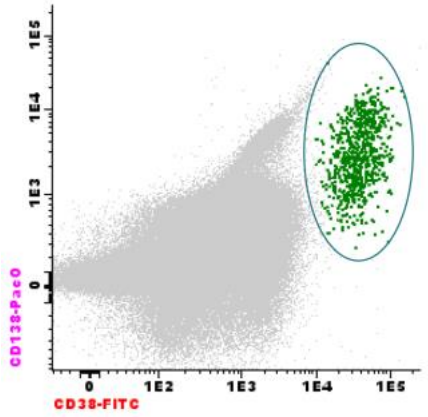
Consideraciones prácticas

morfología



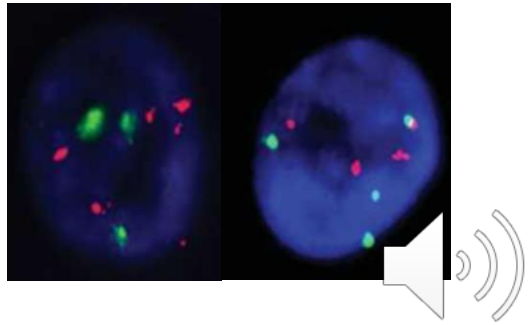
identificación celular





separación celular

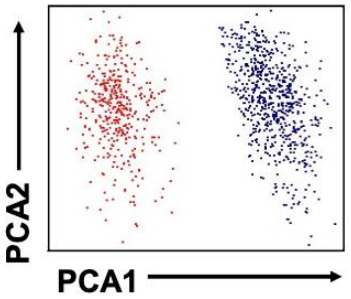
- **FISH**
- **BIOLOGÍA MOLECULAR**
- **SECUENCIACIÓN POR SANGER y NGS**
- **CULTIVO CELULAR**



Mieloma Múltiple

Toma de muestra óptima para un diagnóstico completo

Inmunofenotipo

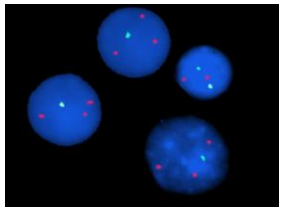


VB421	VB510	FITC	PE	PERCPCY5.5	PE-CY7	APC	APC H7
CD138	CD27	CD38	CD56	CD45	CD19	cyK	cyL
CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81

Cell Sorting

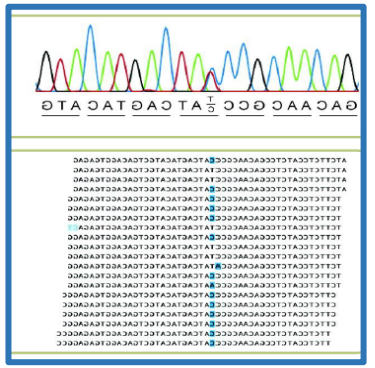
2008 European Myeloma Network report
CD38, CD138, CD45, CD19, CD56, CD27, CD81,

FISH



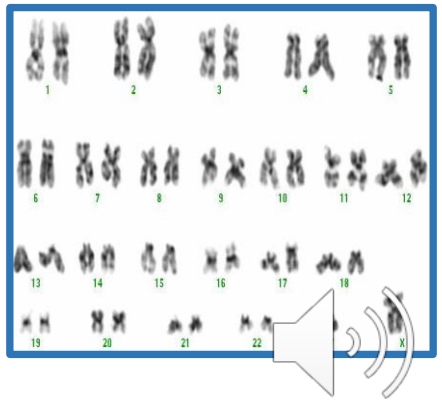
del(17p)
t(4;14)
t(14;16)
1p1q . . .
t(14;20)
t(11:14)

Sanger y NGS



Cariotipo

heparina



Citometría de flujo multiparamétrica

Estandarización del equipo y de procedimientos

Leukemia

Special Report | Open Access | Published: 05 September 2012

EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols

T Kalina, J Flores-Montero, V H J van der Velden, M Martin-Ayuso, S Böttcher, M Ritgen, J Almeida, L Lhermitte, V Asnafi, A Mendonça, R de Tute, M Cullen, L Sedek, M B Vidriales, J J Pérez, J G te Marvelde, E Mejstrikova, O Hrusak, T Szczepański, J J M van Dongen & A Orfao on behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

Leukemia **26**, 1986–2010 (2012) | Download Citation

Estandarización del citómetro de flujo y separador celular con paneles de 8 colores según lineamientos Euroflow

Confiables

Reproducibles

Comparables

- Selección de fluorocromos para paneles de inmunofenotipo con 8 colores
- Settings del citómetro de flujo
- SOP muestras
- Estrategias para el análisis de datos

.

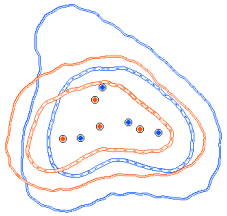
- ✓ Estandarizar las lecturas del BD FACS Cell sorter según los ajustes y lineamientos de EuroFlow

- ✓ Evaluar la correspondencia en ambos instrumentos de las lecturas de Intensidad de Fluorescencia Media (MFI) en muestras normales y patológicas.

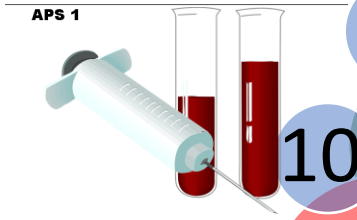
FACS canto	FACS Melody	FACS Aria Fusion
Citómetro de flujo	Cell sorter	Cell sorter
3 láser	3 láser	4 láser
8 fluorescencias	9 fluorescencias	16 fluorescencias
DIVA	FACSChorus	DIVA

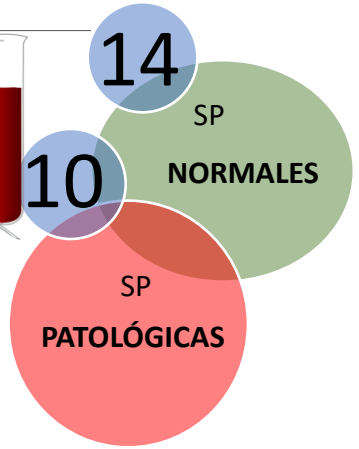
Ajuste diario de la configuración del instrumento

Infinicyt™

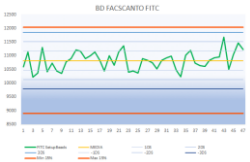


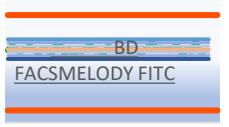
4 Análisis





3 Inmunomarcación



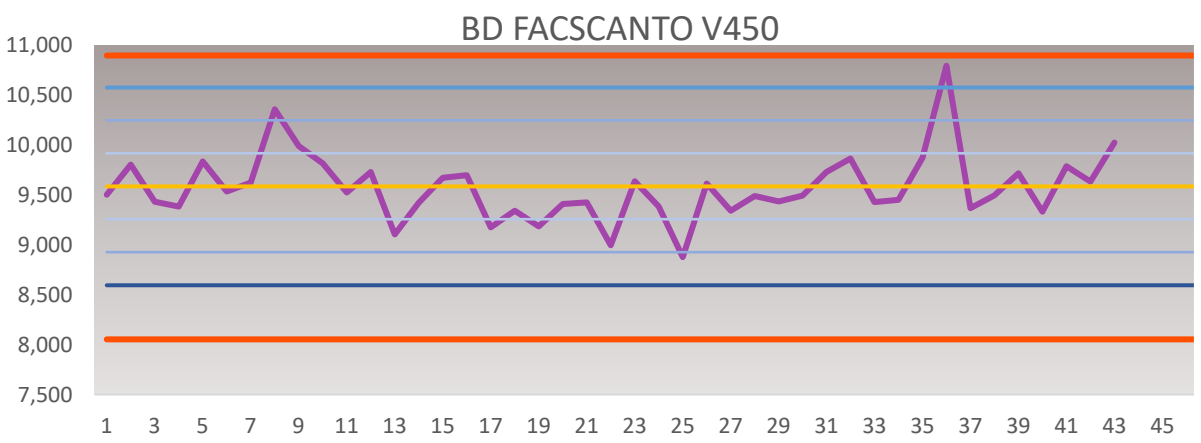


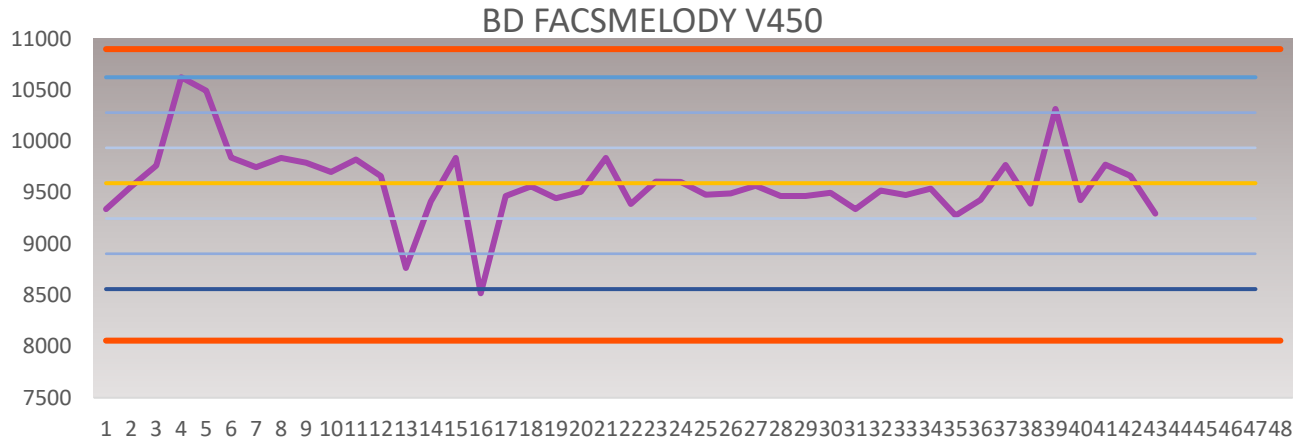
1 6 1116212631364146

2 Lecturas MFI y CV%

1 Cytometer settings

Resultados





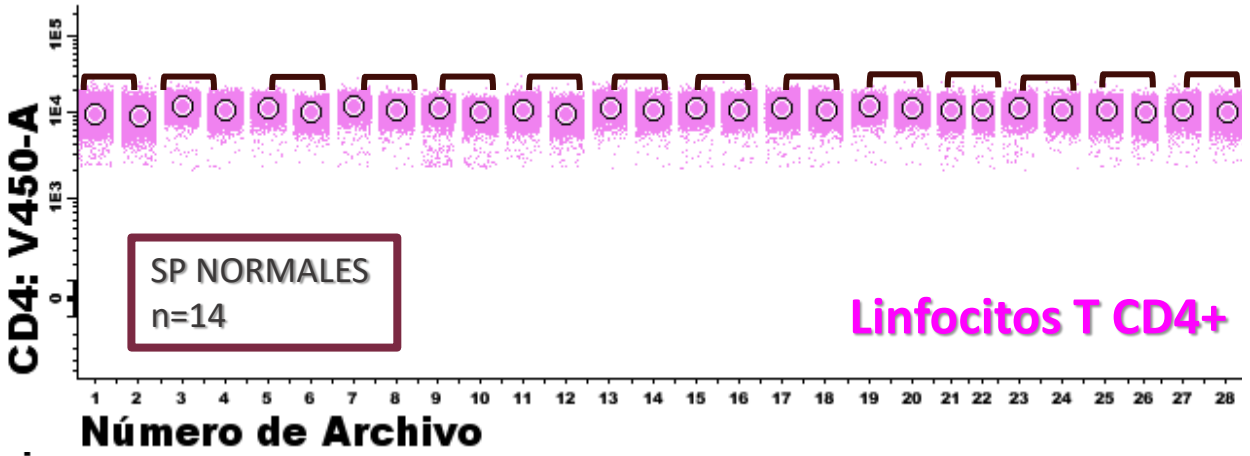
Valor MFI target MFI Límite de estandarización (+/- 15%) Desviación estándar 1,2 y 3

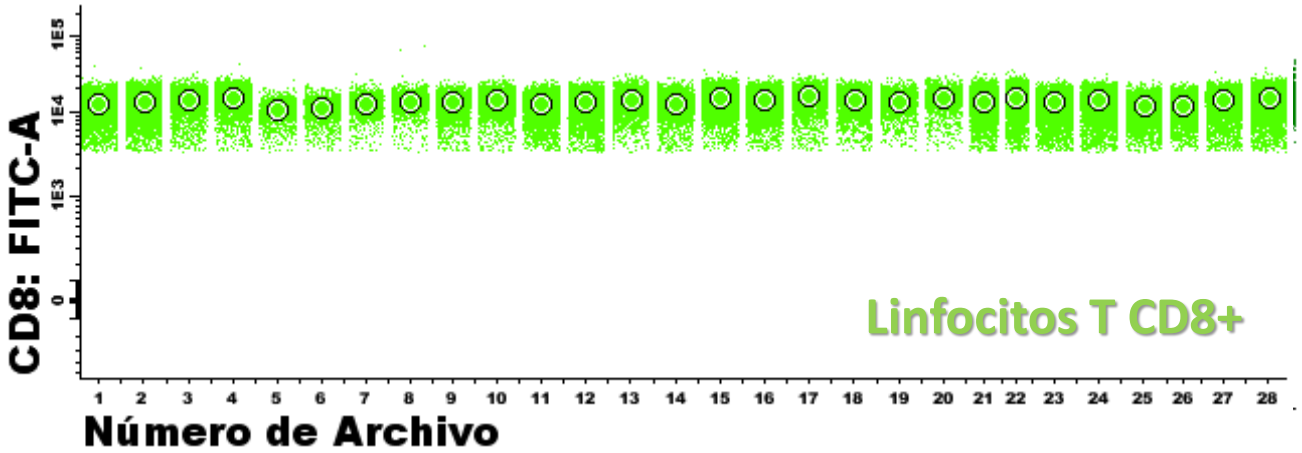
CV% setup beads	FITC	PE	PERCPCY5.5	PECY7	APC	APCH7	V450	V500
FACSCANTO	3.17	3.58	2.92	3.37	4.83	4.60	3.44	3.29
MELODY	0.98	1.29	1.45	1.38	1.91	2.14	3.59	2.06

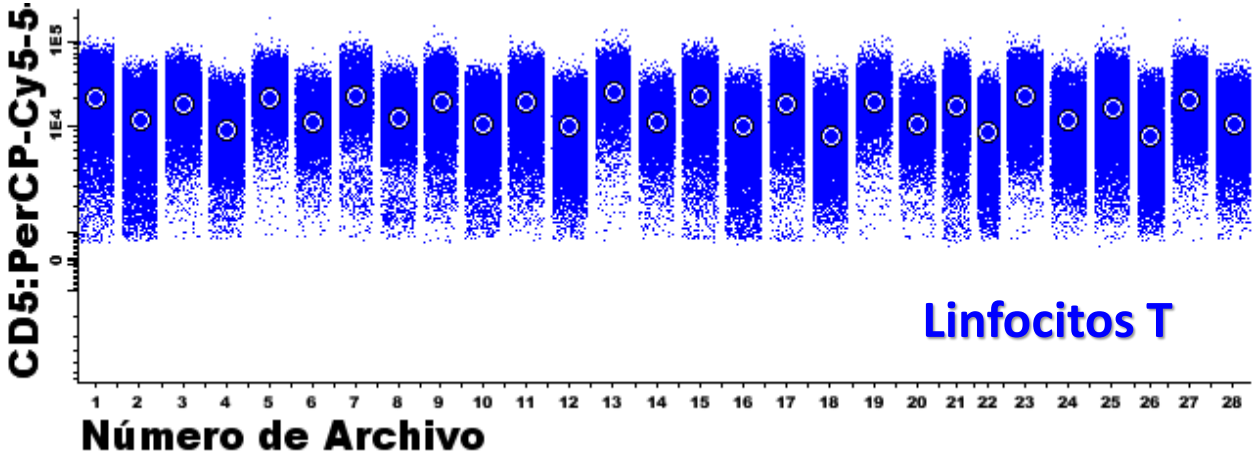
MFI Subpoblaciones linfocitarias en ambos citómetros

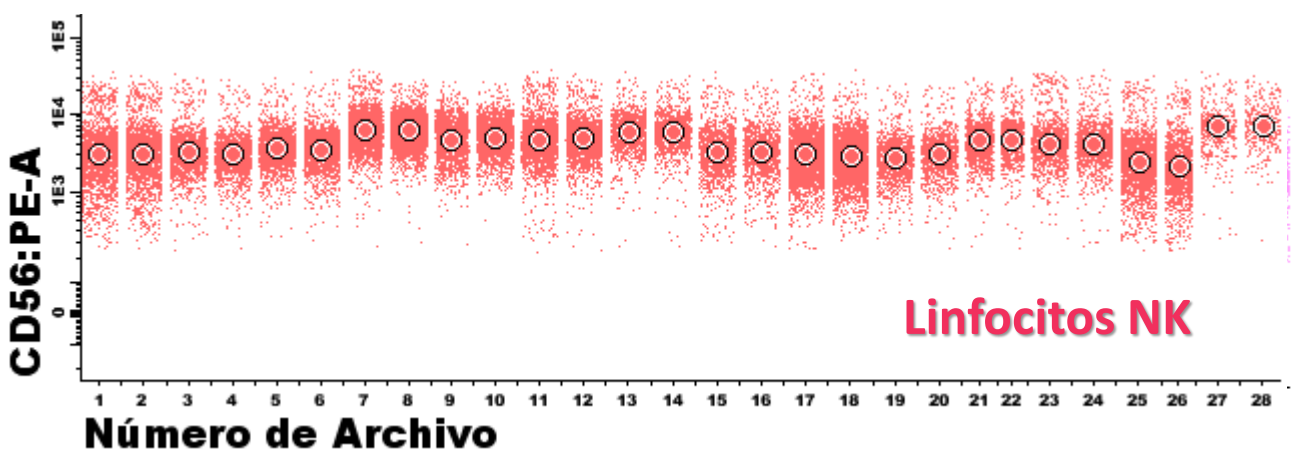
BD FACSCanto II

/ BD FACSMelody





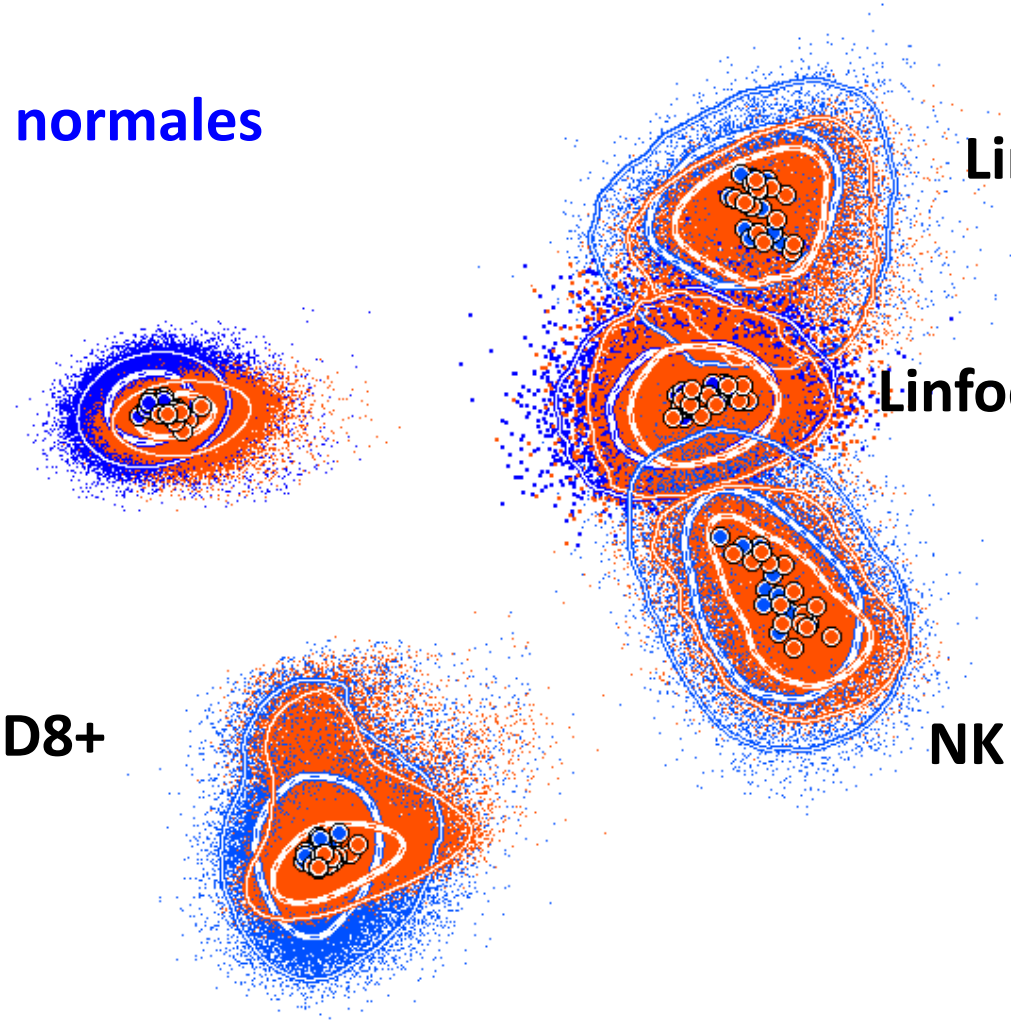




Subpoblaciones linfocitarias normales
FACSort

Subpoblaciones linfocitarias normales
FACSCanto II

SP NORMALES
n=14



Linfocitos T CD4+

Linfocitos B IgK

Linfocitos B IgL

Linfocitos T CD8+

NK Cells

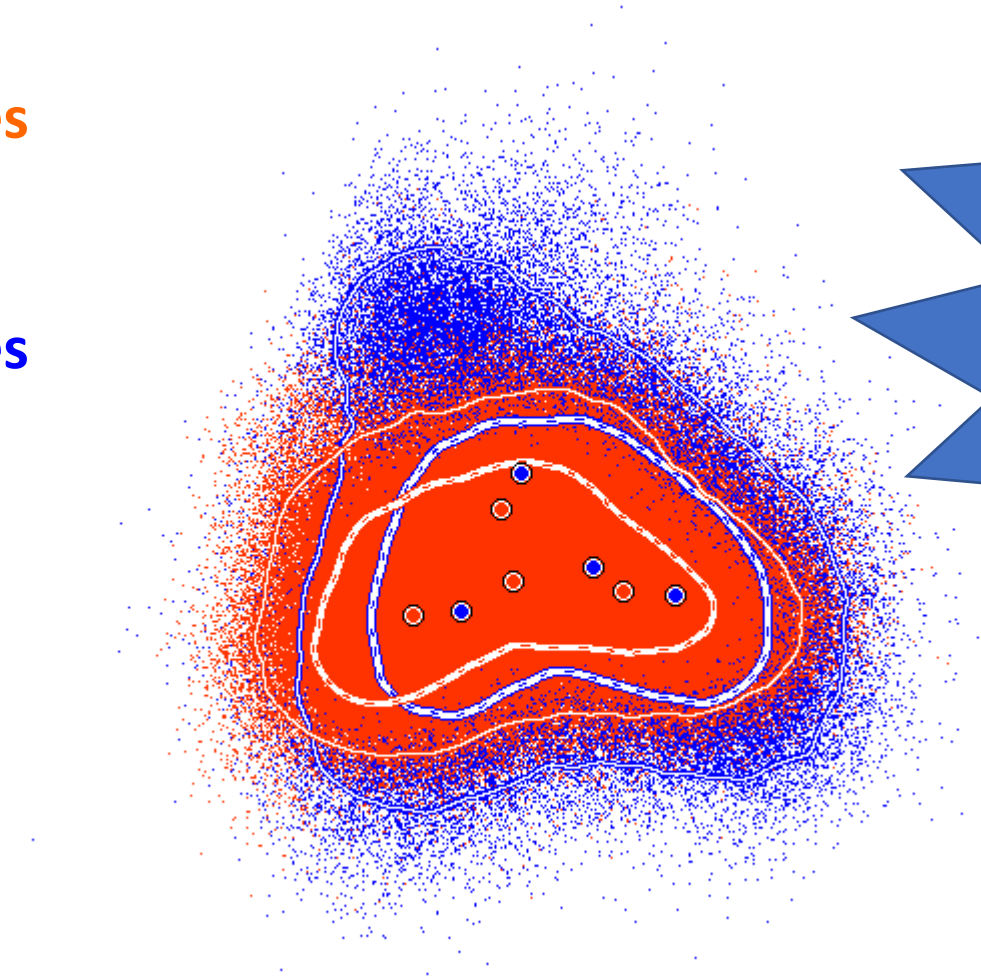
100% de inclusión
en el 1er DS

APS 1

Linfocitos B clonales
LLC - FACSort

Linfocitos B clonales
LLC- FACSCanto II

SP PATOLÓGICAS
n=4



APS 1

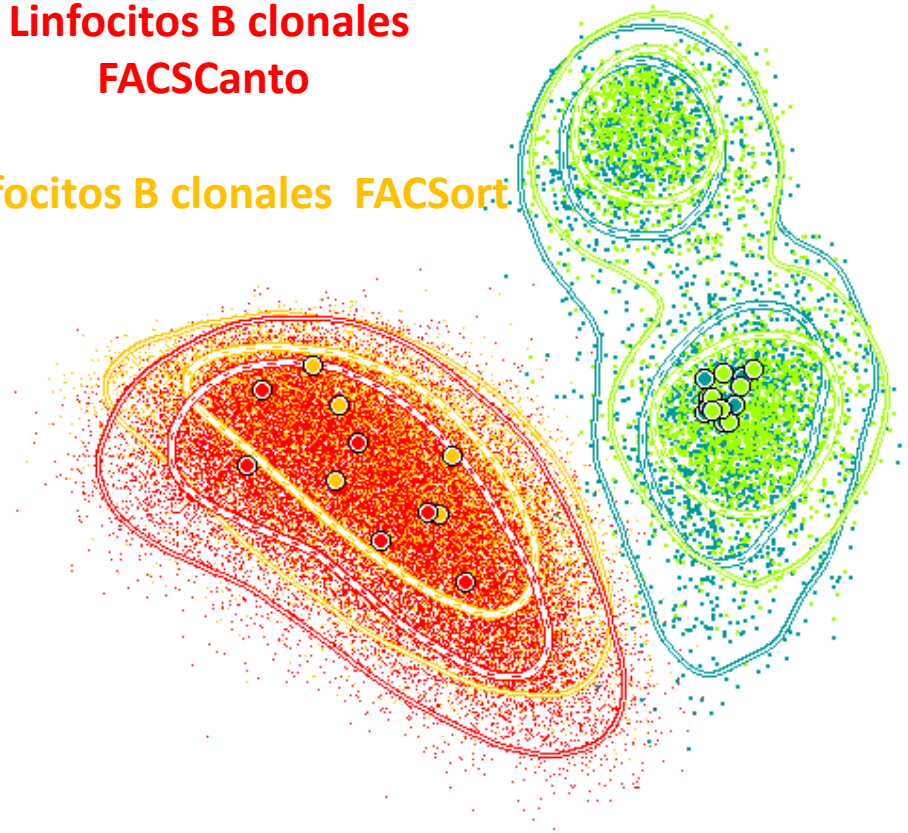
100% de inclusión en el 1er DS

Linfocitos B normales
FACSCanto

Linfocitos B normales FACSort

Linfocitos B clonales
FACSCanto

Linfocitos B clonales FACSort



Información del Diagrama

General | PC1 | PC2

Parámetro	Importancia
V450-A LOGICAL	25.21
CD56+KAPPA:PE-A LOGICAL	16.92
CD19+TCRgd:PE-Cy7-A LOGICAL	14.51
V500-A LOGICAL	8.98
CD23:FITC-A LOGICAL	7.79
FSC-A LINEAL	6.70
FSC-H LINEAL	5.72
APC-H7-A LOGICAL	4.30
SSC-A Exp-SSC Low	3.27
PerCP-Cy5-5-A LOGICAL	2.96
SSC-H Exp-SSC Low	2.84
CD3:APC-A LOGICAL	0.80

General | PC1 | PC2

Parámetro	Importancia
PerCP-Cy5-5-A LOGICAL	27.60
CD23:FITC-A LOGICAL	18.11
CD56+KAPPA:PE-A LOGICAL	17.77
CD3:APC-A LOGICAL	9.83
V450-A LOGICAL	6.43
APC-H7-A LOGICAL	5.81
FSC-A LINEAL	5.48
FSC-H LINEAL	3.35
V500-A LOGICAL	2.27
SSC-A Exp-SSC Low	1.33
CD19+TCRgd:PE-Cy7-A LOGICAL	1.25
SSC-H Exp-SSC Low	0.76

APS 1

APLICACIONES DEL CELL SORTING

(SEPARACIÓN CELULAR)

EN ONCOHEMATOLOGÍA

Evolución de la separación celular

Separador manual
MACS BD
MACS Miltenyi

BD FACSMelody

3 lásers
9 detectores de fluorescencia
2 canales de sorting

BD FACSAria Fusion

4 lásers
15 detectores de fluorescencia
4 canales de sorting

Aplicaciones De La Citometría De Flujo Multiparamétrica Y Sorting De Alta Velocidad

BD FACSAria Fusion

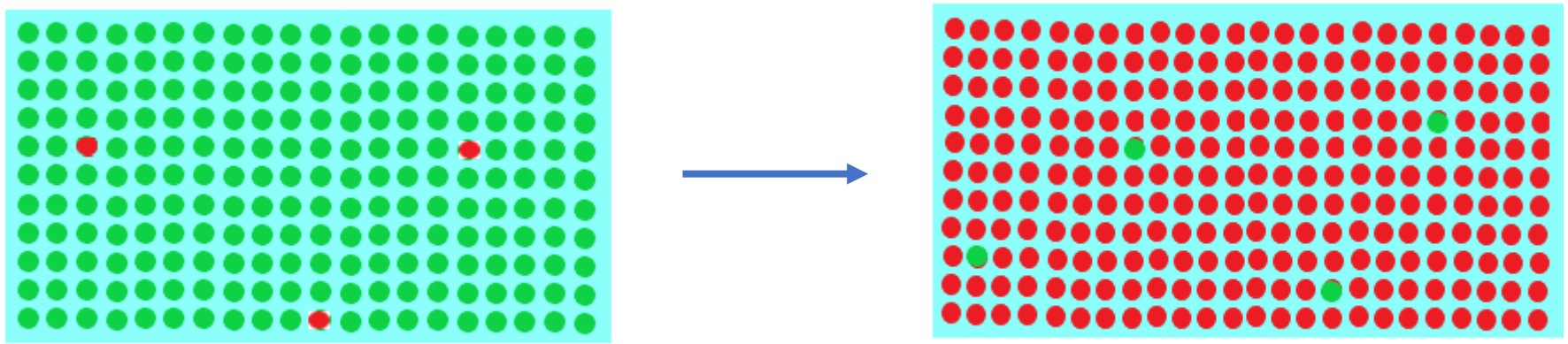
High-performance analysis, high-performance sorting

The fusion of safety, performance, and sorting

BD FACSAria Fusion has precisely integrated fluidic and optical systems to maximize signal detection.
Optimized fiber-launched lasers improve sensitivity and resolution for each color in a multicolor assay,
The flow cell with gel-coupled cuvette ensure performance, safety, and ease-of-use.
Class II Type A2 biosafety cabinet

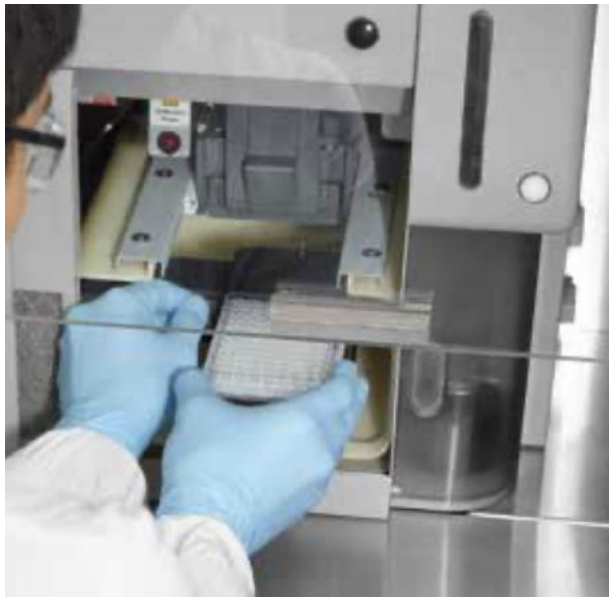
La sensibilidad de las técnicas utilizadas en la detección de alteraciones genéticas puede verse limitada cuando la población neoplásica se encuentra escasamente representada.

Enriquecer la población patológica por Cell Sorting incrementa la sensibilidad en la detección de marcadores genéticos en estos pacientes.



Opciones de colección del post sort

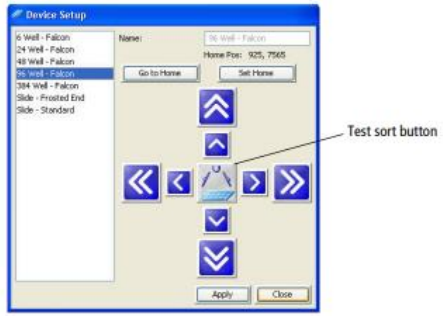
BD FACSAria Fusion

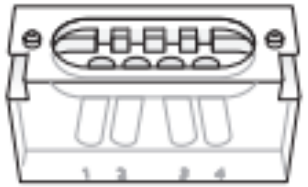


It is designed to sort into

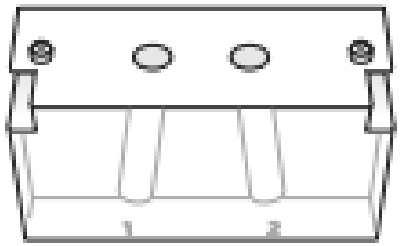
- 5ml FACS tubes,
- 15ml conical tubes
- Eppendorf tubes,
- plates ranging from 6, 12, 48, 96, or 384 Well Plates (Culture or PCR)
- slides microscopia
- petri dishes

Sort collection devices

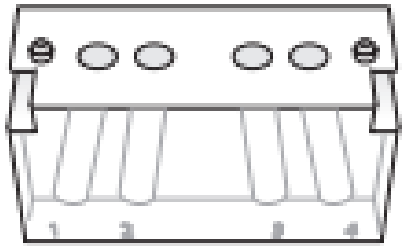




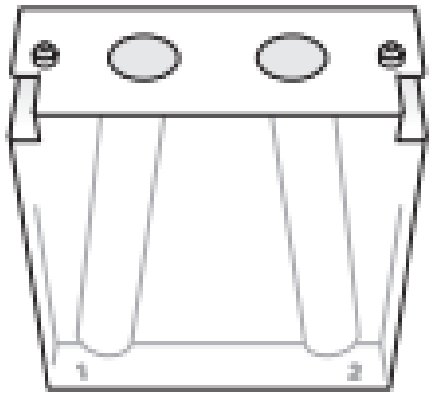
Four-way 1.5mL Eppendorf



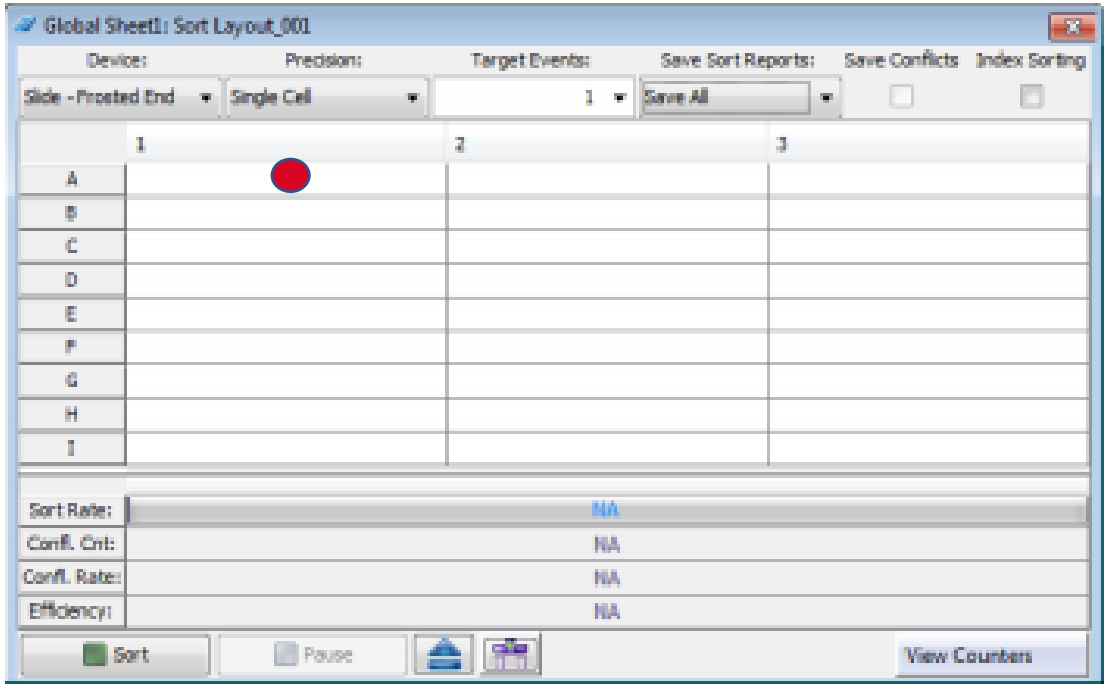
Two-way 12x75mm FACS tubes

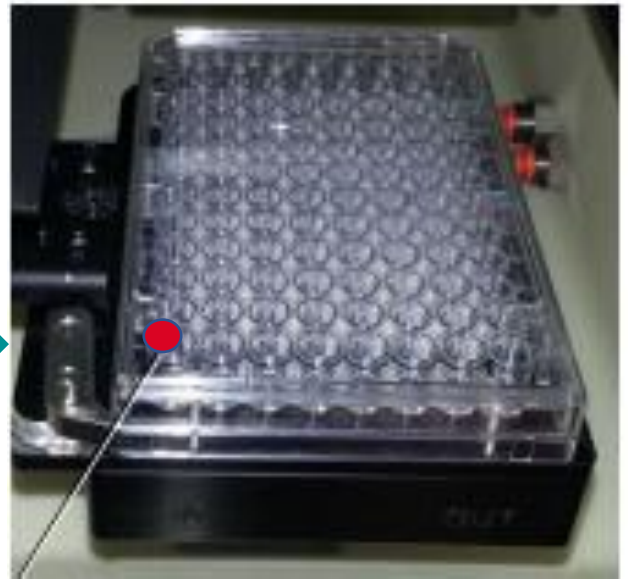


Four-way 12x75mm FACS tubes

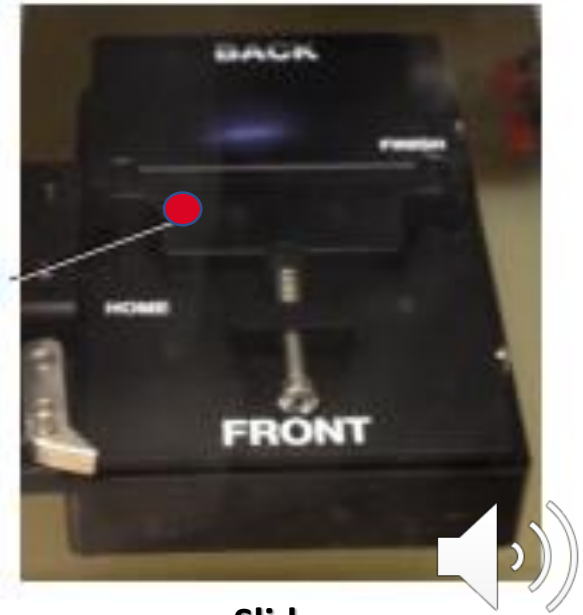


Two-way 15mL Conical tubes





Plate



Slide

Solución de recolección del post sort

- Solución fisiológica
- PBS con BSA / PBS sin BSA (biología molecular)
- RPMI con BSA
- MeOH : AcH (3:1), solucion de Carnoy

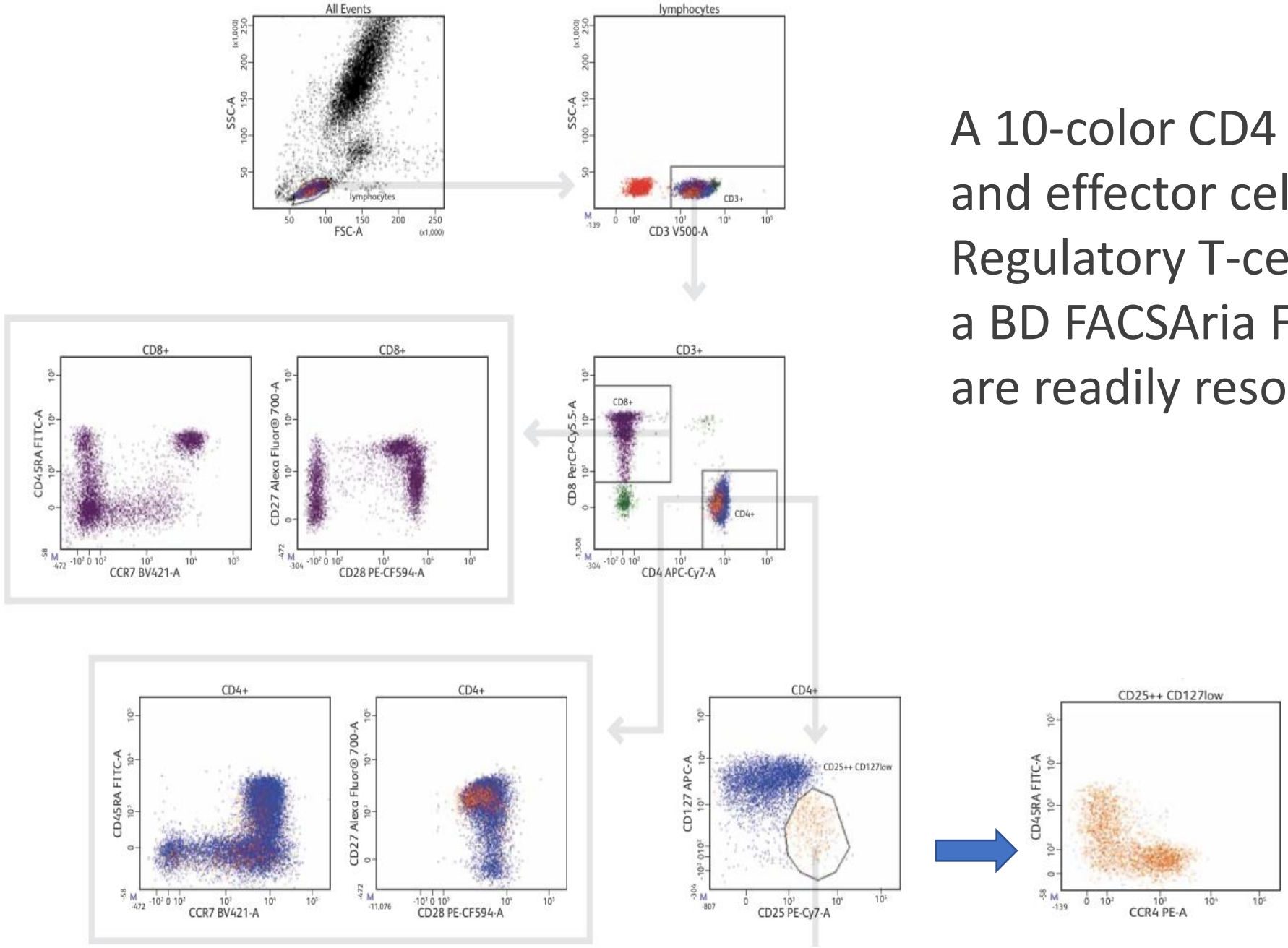
Cantidad de células óptimas para cada técnica

- FISH 5.000 células por FISH.
- Mutación puntual

  BRAF, >50.000 células de interés

  L265P del gen MYD88: >50.000 células de interés.
- Clonalidad B y T: >50.000
- Secuenciación por Sanger:

  mutaciones en p53 en LLC: >50.000
- Test de HUMARA: >15.000 células por población celular
- NGS: >3.500.000/20ng DNA

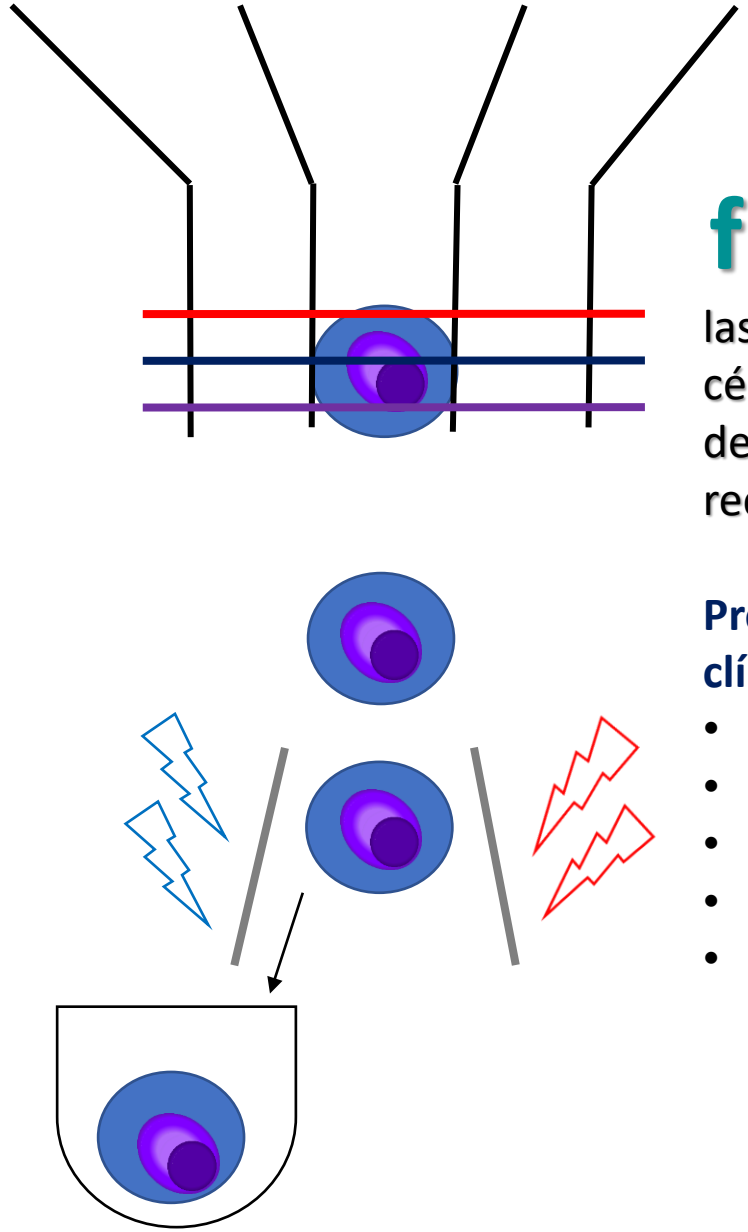


A 10-color CD4 and CD8 memory and effector cell panel and a Regulatory T-cell panel were run on a BD FACSAria Fusion. T-cell subsets are readily resolved.

CELL SORTING

Proceso de separación física de células o partículas en base a la expresión diferencial de parámetros analizables:

- tamaño, complejidad interna, fluorescencias
- por campo electromagnético de alta potencia en un citómetro de flujo.



fundamento

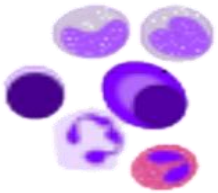
las gotas que contienen la célula de interés se cargan y deflectan al tubo de recolección.

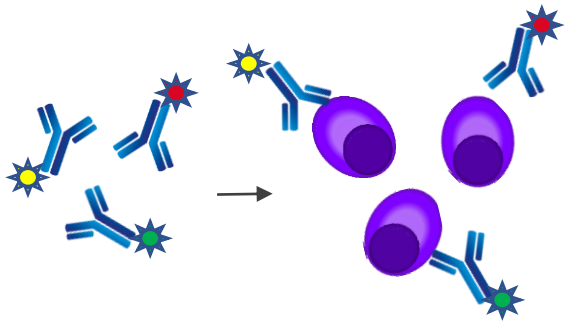
Productos del sorting en clínica:

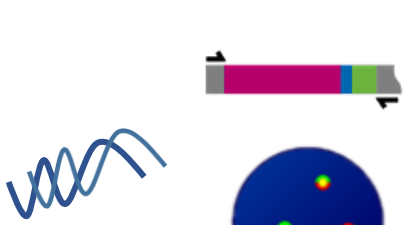
- FISH,
- Rearreglos de TCR y BCR,
- test de Humara,
- Mutaciones puntuales,
- NGS, entre otros.

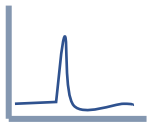
MATERIALES Y MÉTODOS

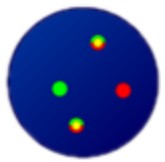
n=31
28 MO: 27MM, 1LLAB
3 SP: Expansiones LT











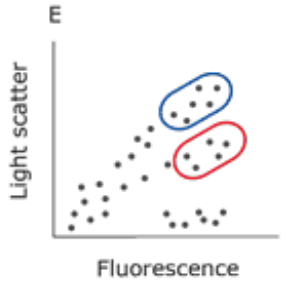
INMUNOMARCACIÓN

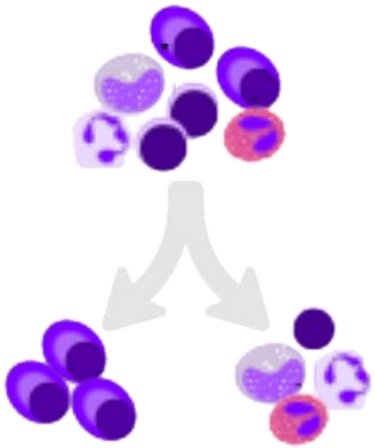
DETECCIÓN DE PATOLOGÍAS ONCOHEMATOLOGICAS ESCASAMENTE REPRESENTADAS

BULK LISIS E INMUNOMARCACIÓN

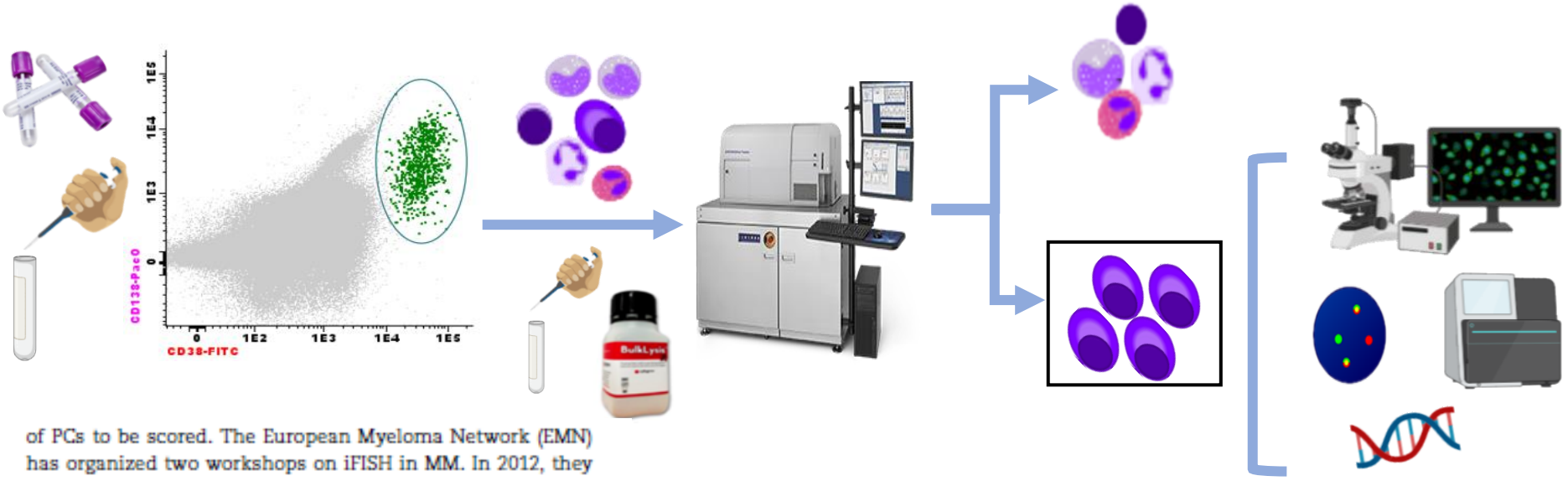
SEPARACIÓN CELULAR

FISH
REARREGLOS DEL TCR Y BCR
TEST DE HUMARA
MUTACIONES PUNTUALES
NEXT GENERATION SEQUENCING





Protocolo de trabajo – Cell Sorting



of PCs to be scored. The European Myeloma Network (EMN) has organized two workshops on iFISH in MM. In 2012, they published some technical recommendations herein transcribed from the paper:

(1) Material should be part of the first draw of the aspirate;
(2) Samples should be sent at suitable times to allow for the lengthy processing procedure;
(3) Most importantly, PCs must be purified or specifically identified;
(4) Cut-off levels should be relatively conservative: 10% for fusion or breakapart probes, and 20% for numerical abnormalities;
(5) Informative probes should be combined for best effect;
(6) In specialist laboratories, a single experienced analyst is considered adequate;
(7) At least 100 PCs should be scored;
(8) Essential abnormalities to test for are t(4;14), t(14;16) and 17p13 deletions;
(9) Suitable commercial probes should be available for clinically relevant abnormalities;
(10) The clinical report should be expressed clearly and must state the percentage of PC involved and the method used for identification".[4]

Separación de células de interés con una **concentración al menos del 95%**
Necesaria para la correcta evaluación de factores pronósticos por FISH

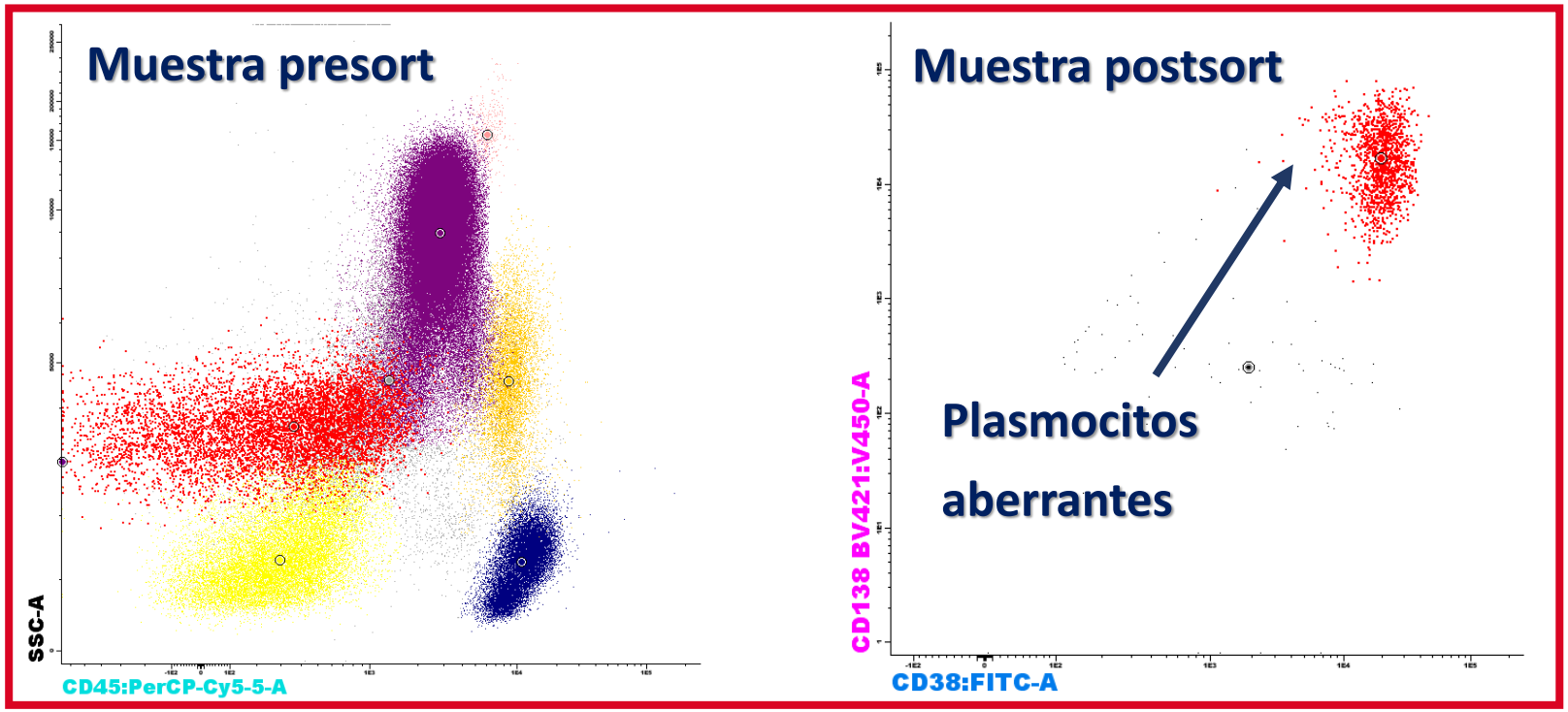
Utilidad de la separación celular ¨cell sorting¨ en el diagnóstico clínico

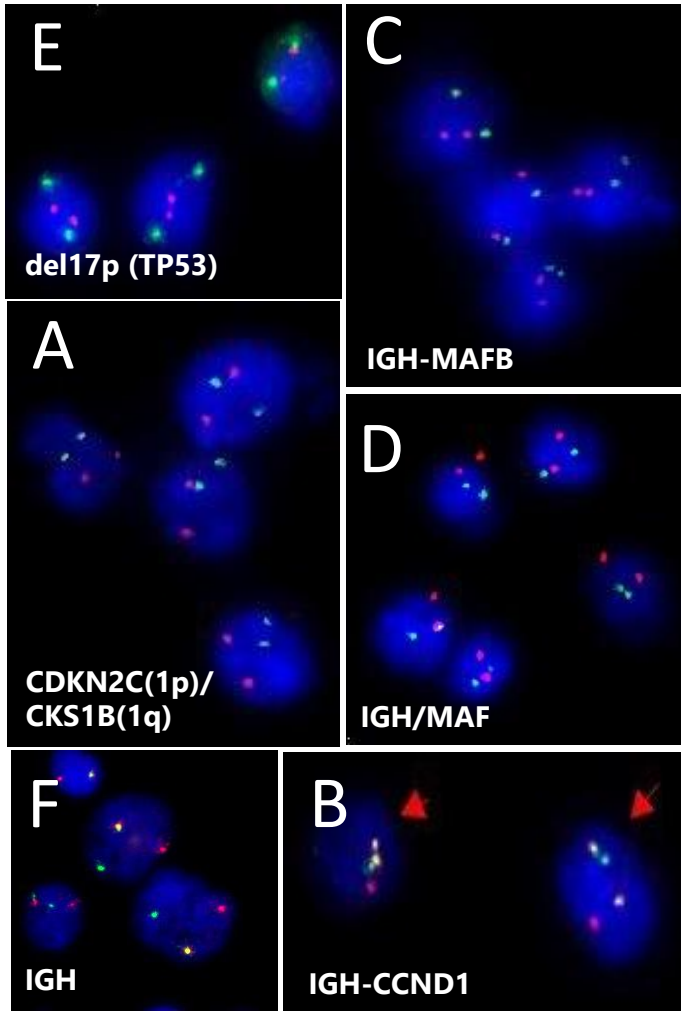
FISH en Mieloma Múltiple al diagnóstico

pronóstico y opciones terapéuticas

RESULTADOS

- ✓ MM (n=27). Sort count: 100.000 plasmocitos.
- ✓ MM: 5% ➡ 95%.
- ✓ Las muestras procesadas para FISH se analizaron e interpretaron correctamente.





Caracterización citogenética en muestra sorteada de mieloma múltiple por FISH (A); (C); (D) y (E) patrón normal para CDKN2C(1p)/CKS1B(1q), IGH-MAFB, IGH-MAF y del17p (TP53). (B) IGH-CCND1 patrón rearreglado (F) IGH rearreglado

Utilidad de la separación celular ¨cell sorting¨ en el diagnóstico clínico

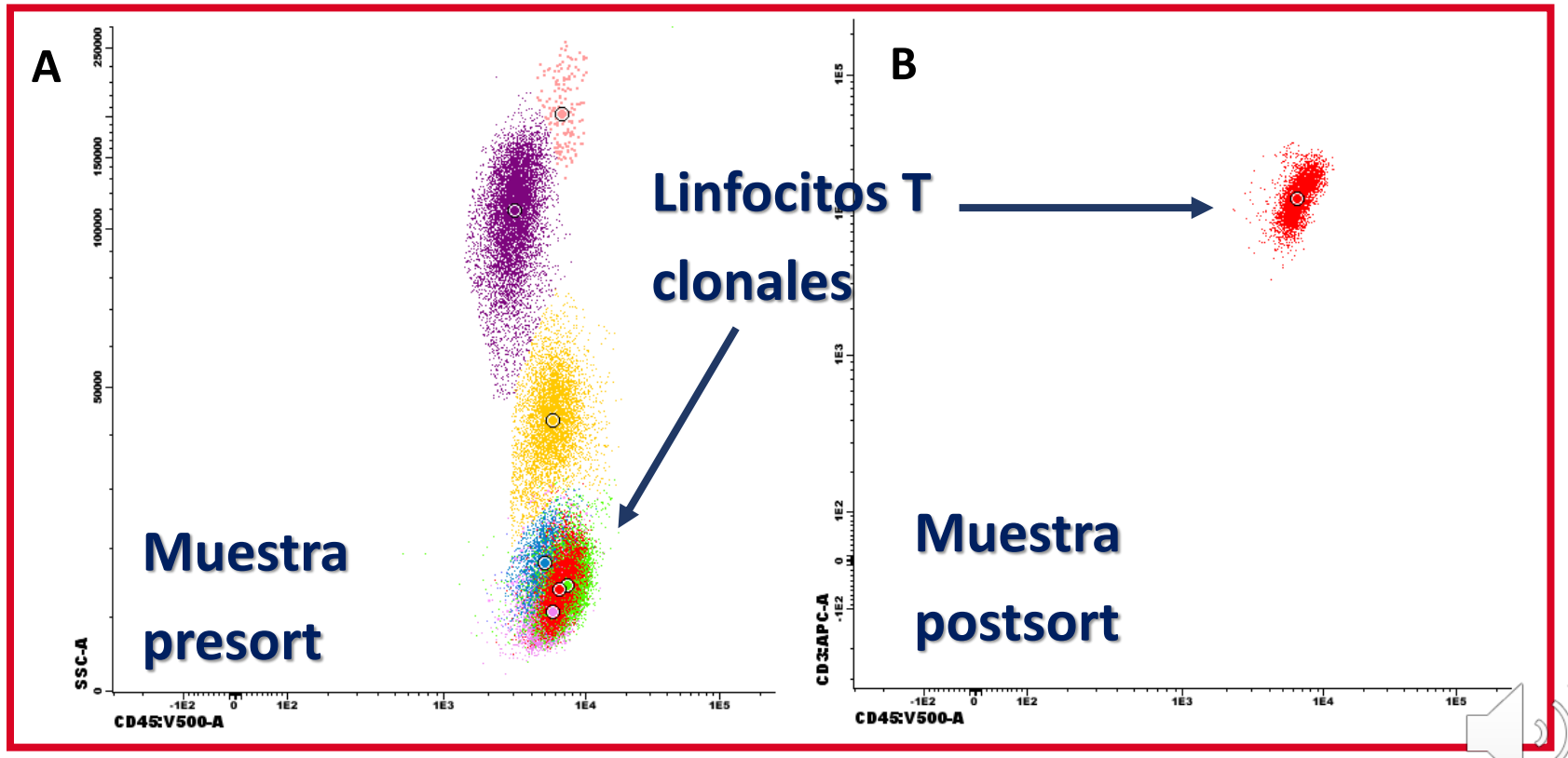
Evaluación del Rearreglo del TCR

en expansiones de linfocitos aberrantes

Estudio de clonalidad de linfocitos T

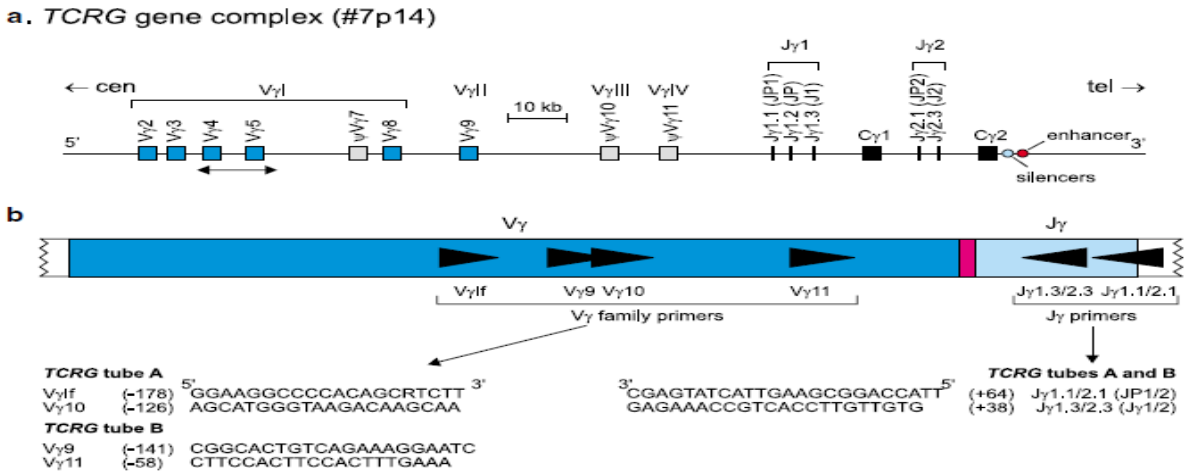
- ✓ 3 expansiones T.
- ✓ Sort count: 50.000 linfocitos T.

Presort		Postsort
✓ 4%	➡	95%
✓ 9%	➡	98%
✓ 20%	➡	99%

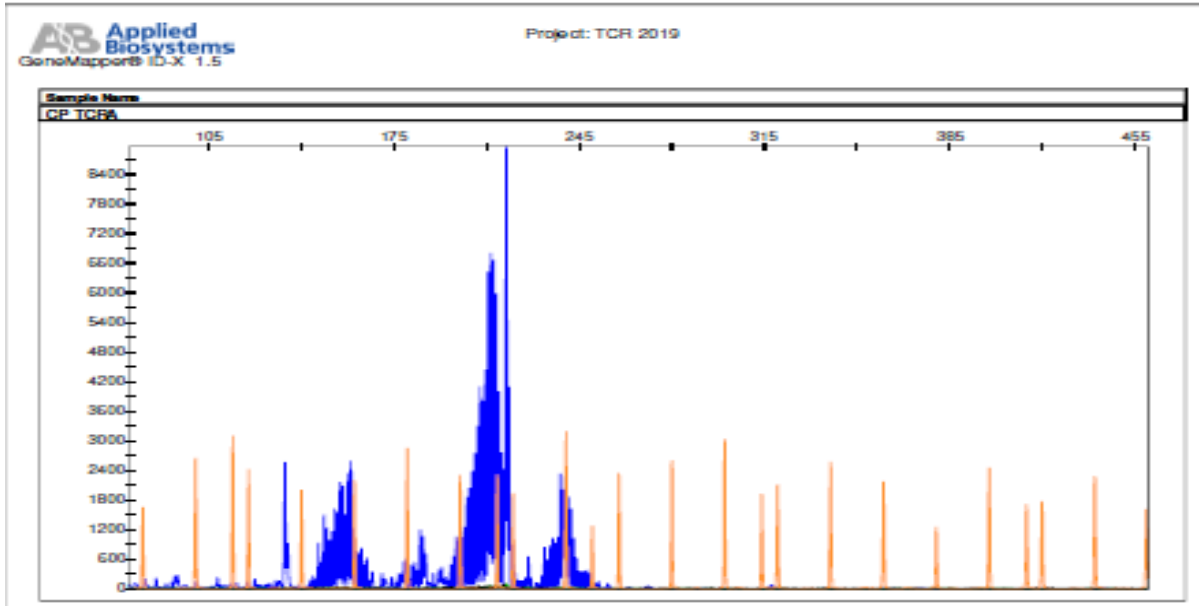


Clonalidad de Linfocitos T

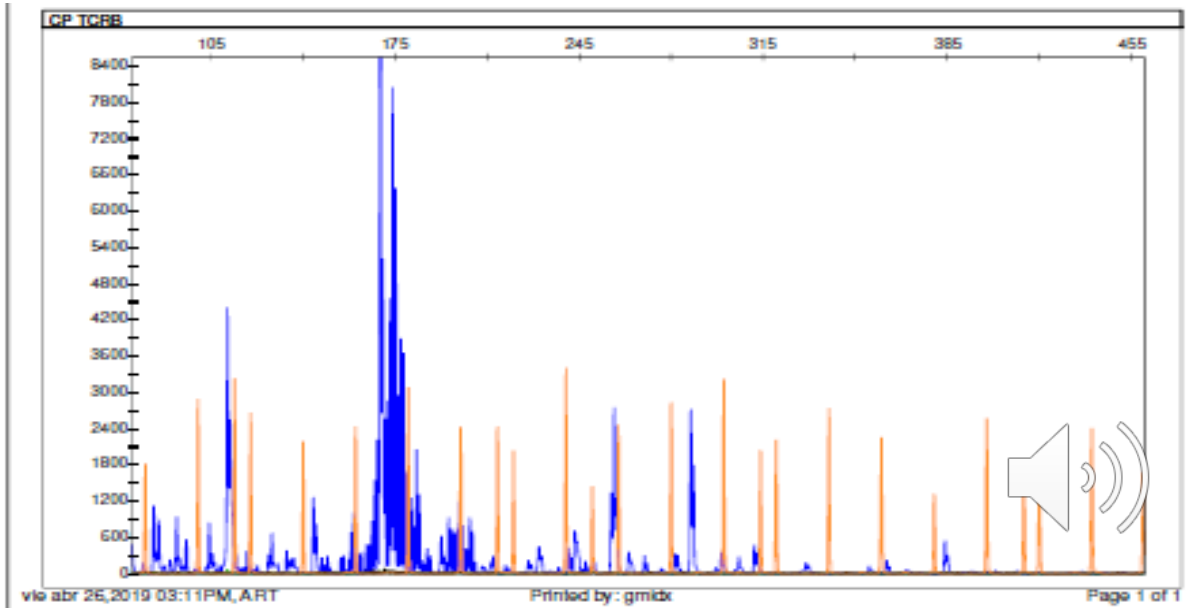
Metodología: A partir del material remitido se extrajo ADN y se realizó amplificación por PCR (reacción en cadena de la polimerasa) de los segmentos reordenados VJ del gen de la cadena gamma del receptor de linfocitos T (TCRgamma).
La detección del reordenamiento se realiza mediante GeneScan (electroforesis capilar) y para su interpretación se utiliza el software Gene Mapper ID-X.



Mix A



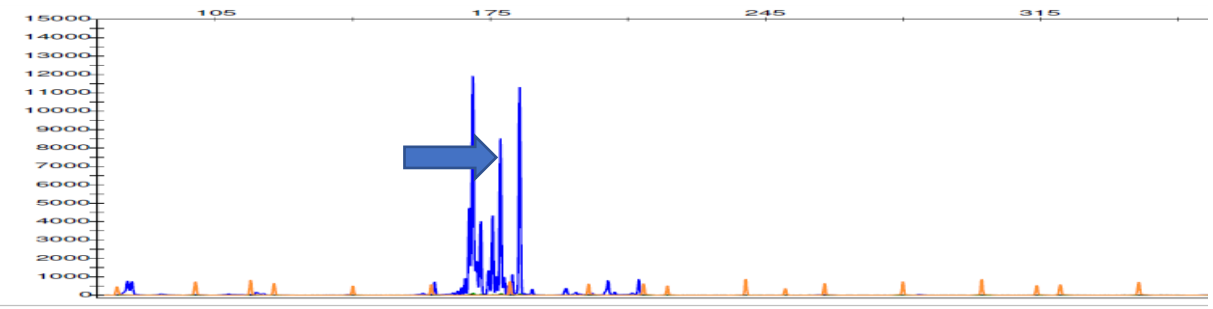
Mix B



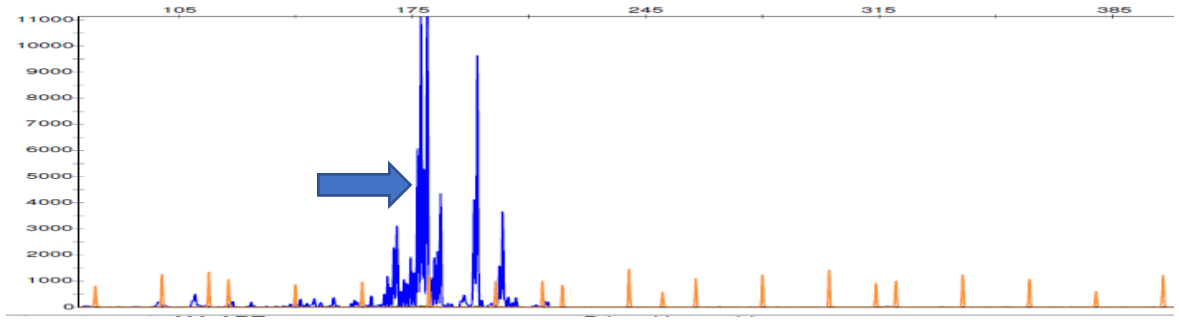
Clonalidad de linfocitos T por análisis de fragmentos

Presort sangre entera

Mix A

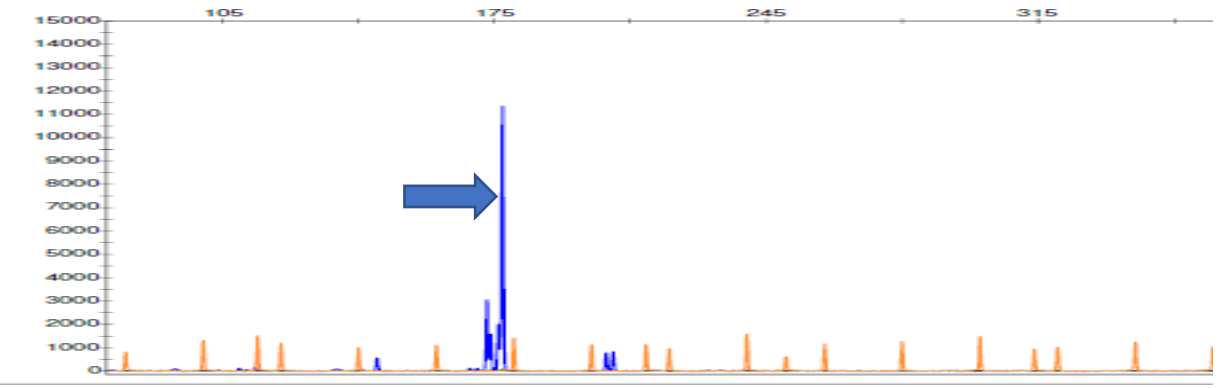


Mix B

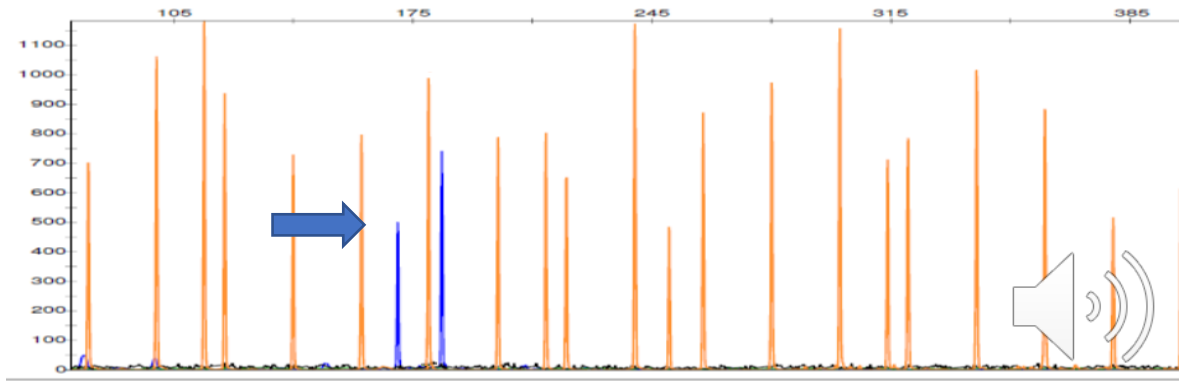


Postsort

Mix A



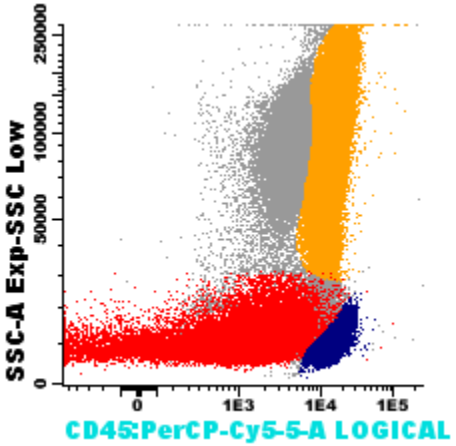
Mix B

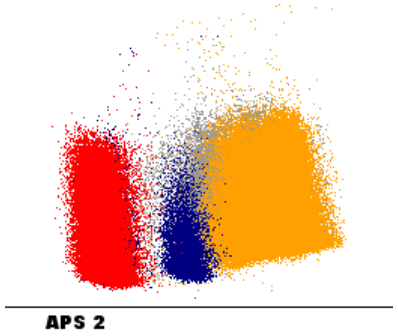


Utilidad de la separación celular ¨cell sorting¨ en el diagnóstico clínico

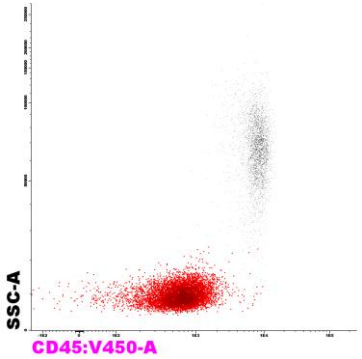
FISH en linfoblastos de Leucemia Linfoblástica B

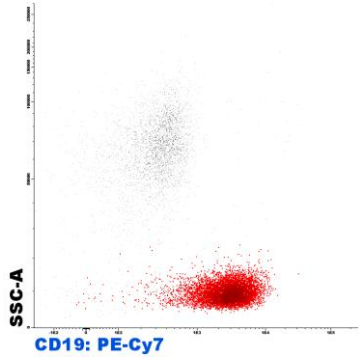
con componente eosinofílico

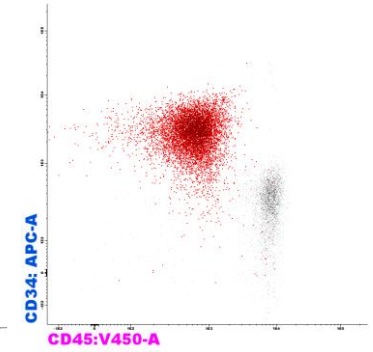




LLA B con eosinofilia

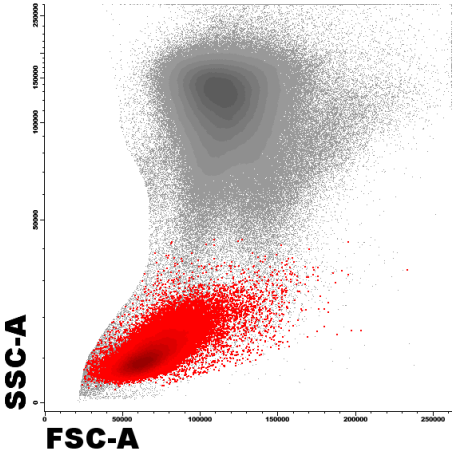






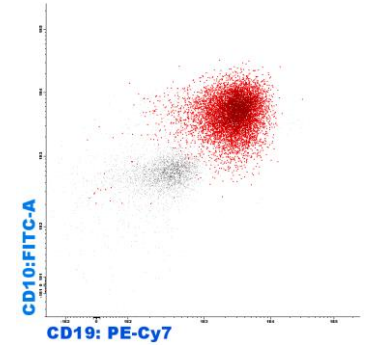
Pre Sort

7% linfoblastos B
85% eosinófilos

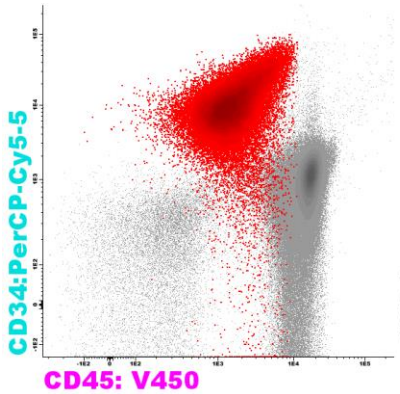


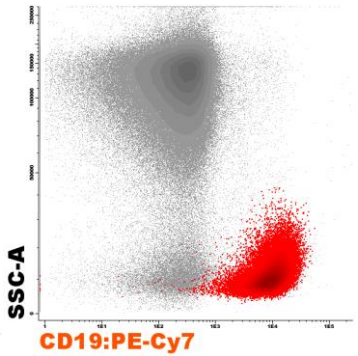
B-lymphoblastic leukaemia/lymphoma with t(5;14)(q31.1;q32.1); IGH/IL3

Post Sort
87% PUREZA



OBJETIVO → RECUPERACION





- blasts with translocation between *IL3* and an IGH gene, resulting in variable eosinophilia.
- immunophenotypic and genetic findings even if the bone marrow blast count is low.
- blasts may be absent in the peripheral blood.
- the *IL3* gene on chromosome 5 and an IGH gene on chromosome 14, resulting in constitutive overexpression of IL3

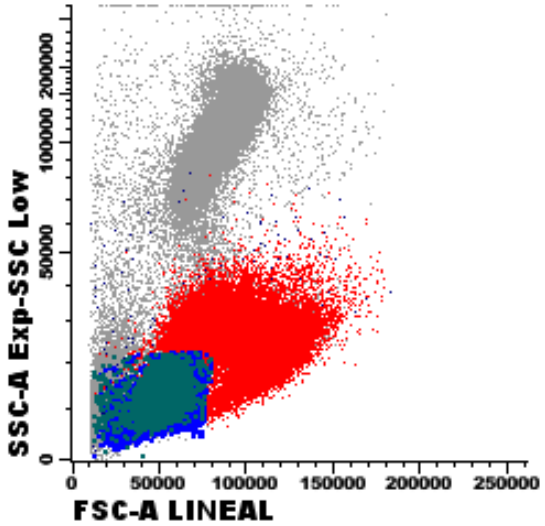
Utilidad de la separación celular ¨cell sorting¨ en el diagnóstico clínico

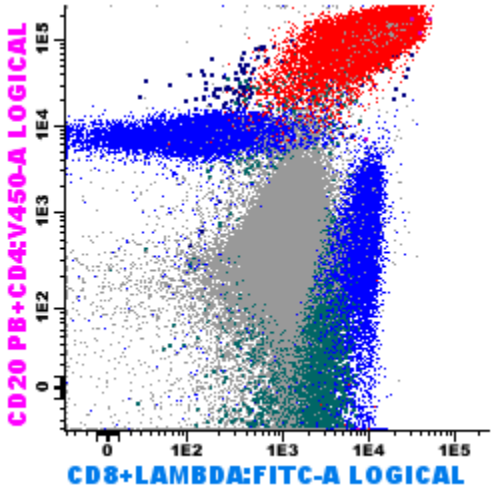
Detección de BRAF en recaída de

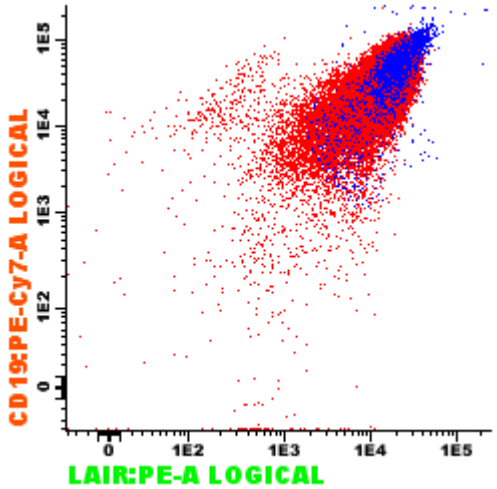
Leucemia de células Vellosas

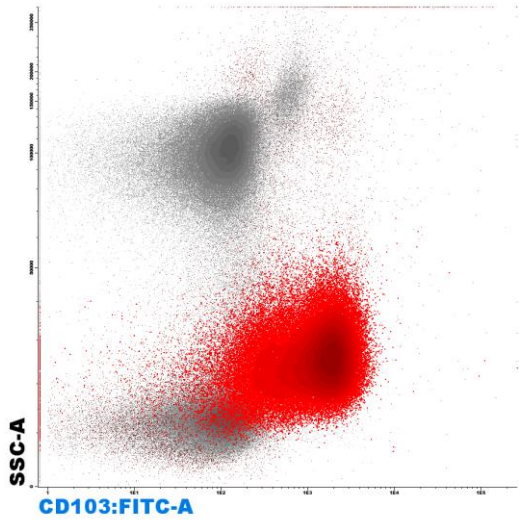
Recaída: células B monoclonales 0,3%

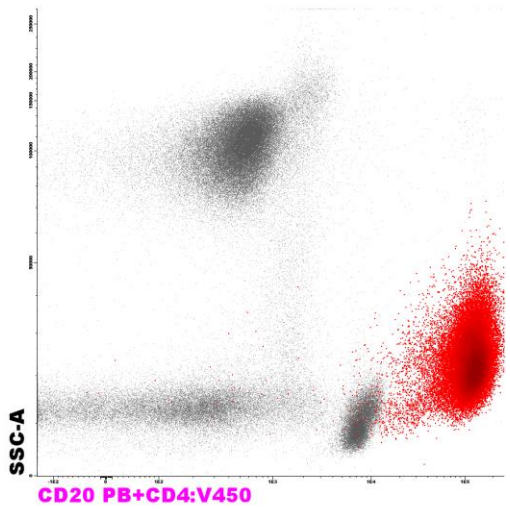
Pre Sort

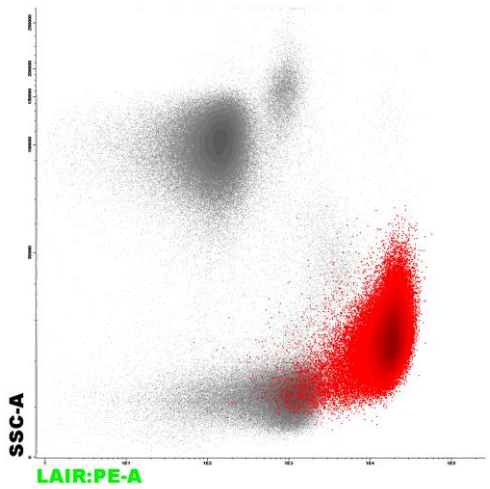






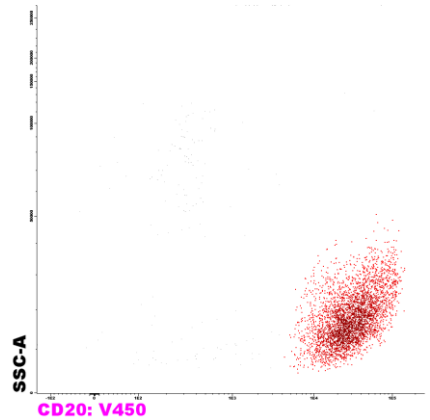


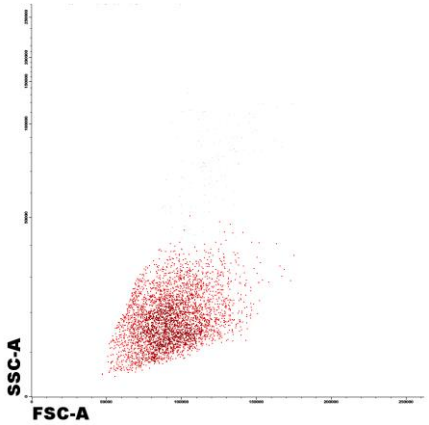




Post Sort 47000 células,

94% pureza, sorting por rendimiento,

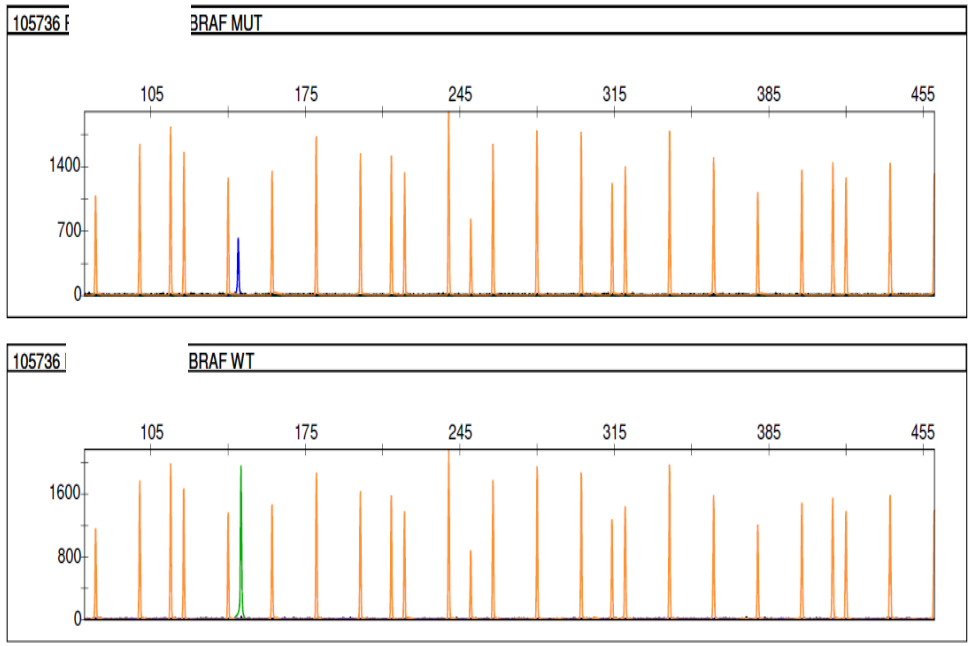




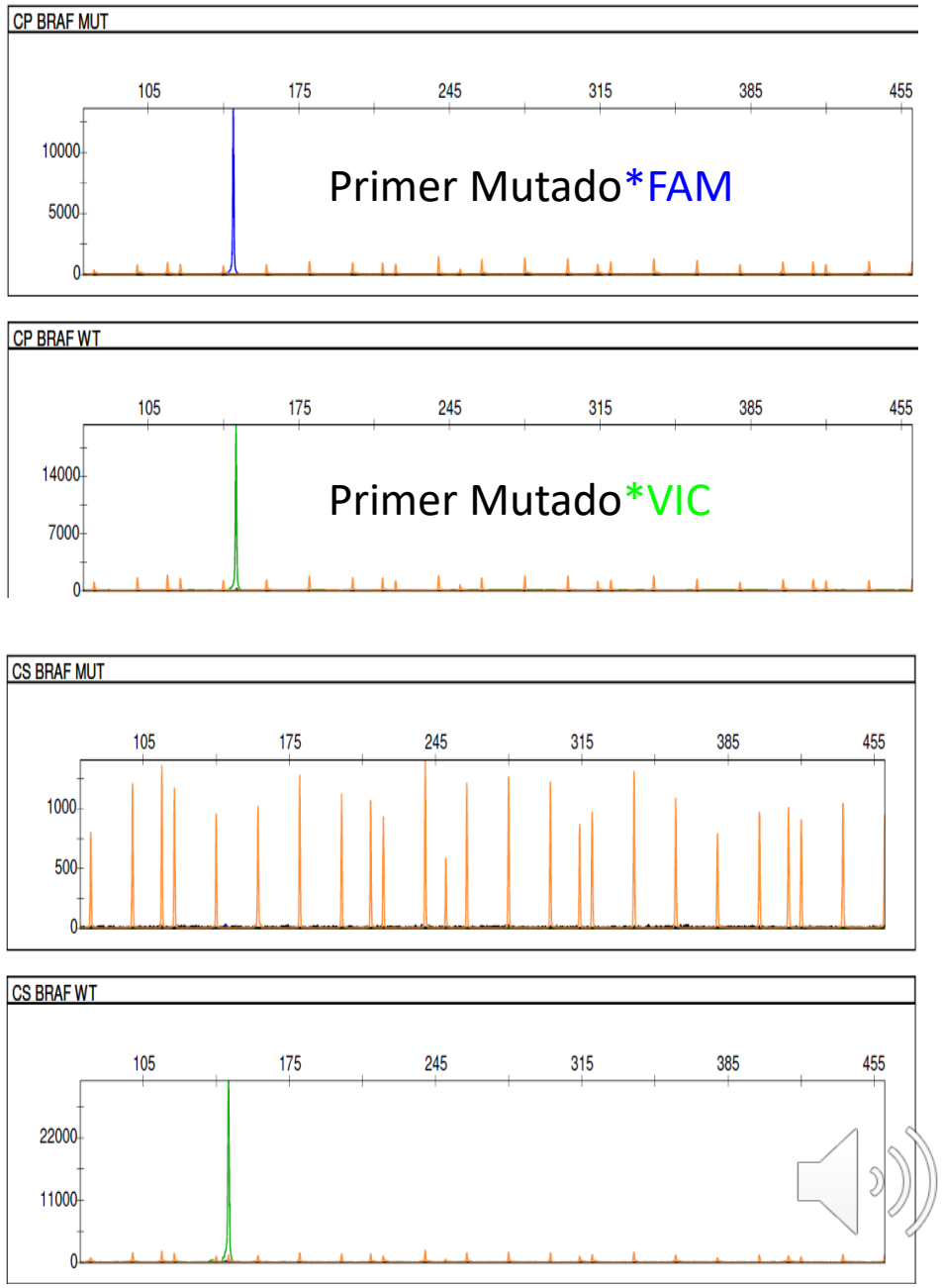
Separación de ADN con columnas

Hairy cell leukemia

BRAF



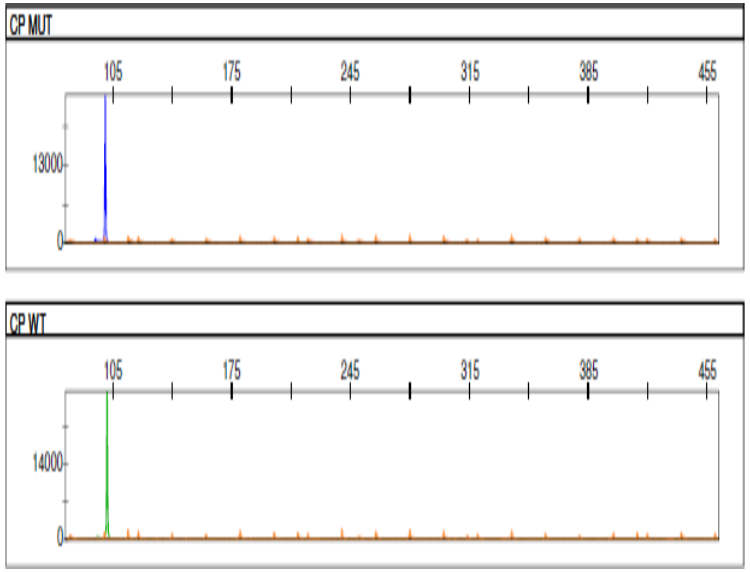
BRAF PCR-alelo específica- tamaño del producto 292pb

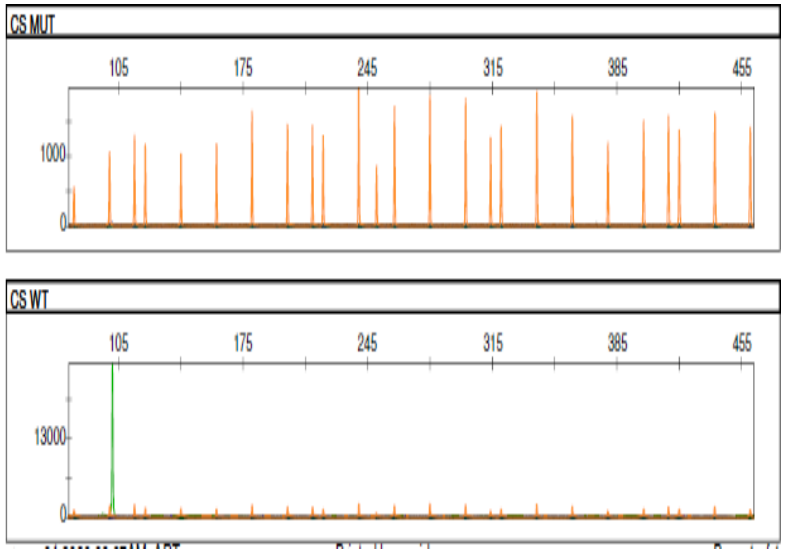


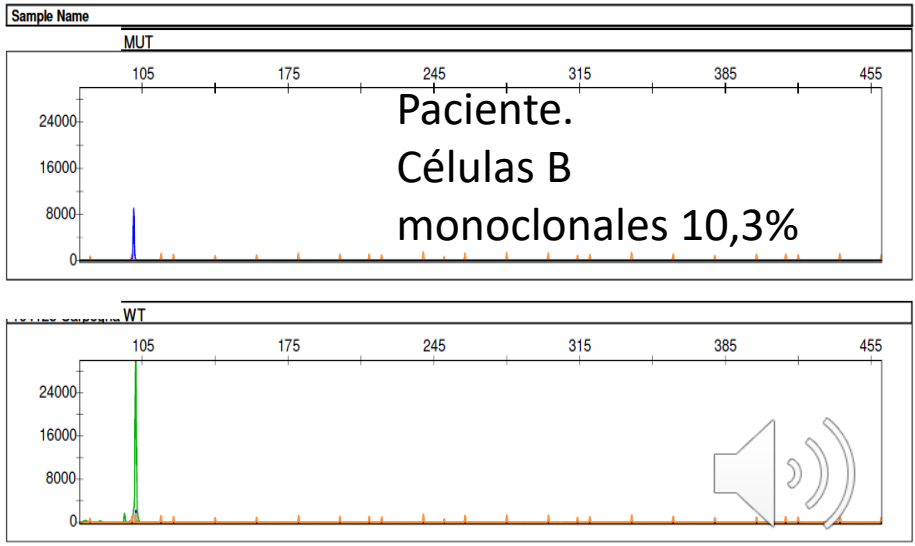
Linfoma linfoplasmocítico Myd88

- Recaída: Pre Sort células B monoclonales 0,2%
- Post Sort 78000 células, 87% pureza,
- sorting por rendimiento,
- Separación de ADN con columnas

MYD-88 PCR-alelo específica - tamaño del producto 102pb







Nuevas entidades: sorting para FISH y secuenciación

A high-risk, Double-Hit, group of newly diagnosed myeloma identified by genomic analysis

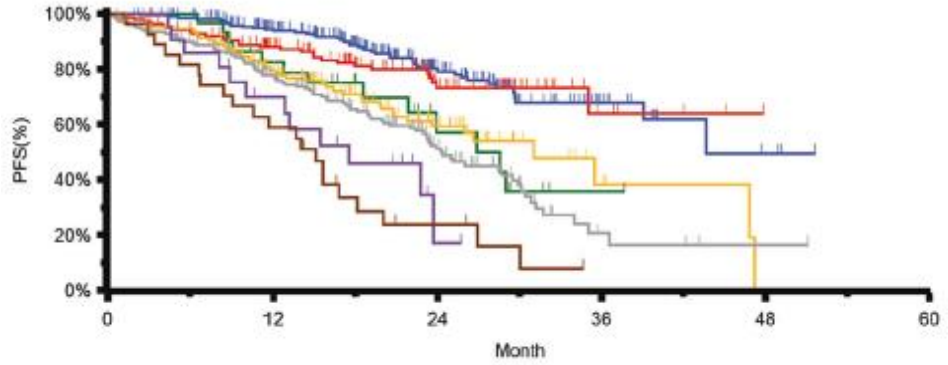
Brian A. Walker[1] · Konstantinos Mavrommatis[2] · Christopher P. Wardell[1] · T. Cody Ashby[1] · Michael Bauer[1] · Faith Davies[1] · Adam Rosenthal[3] · Hongwei Wang[3] · Pingping Qu[3] · Antje Hoering[3] · Mehmet Samur[4] · Fadi Towfic[5] · Maria Ortiz[6] · Erin Flynt[5] · Zhinuan Yu[5] · Zhihong Yang[5] · Dan Rozelle[7] · John Obenauer[7] · Matthew Trotter[6] · Daniel Auclair[8] · Jonathan Keats[9] · Niccolo Bolli[10] · Mariateresa Fulciniti[4] · Raphael Szalat[4] · Phillipe Moreau[11] · Brian Durie[12] · A. Keith Stewart[13] · Hartmut Goldschmidt[14] · Marc S. Raab[14,15] · Hermann Einsele[16] · Pieter Sonneveld[17] · Jesus San Miguel[18] · Sagar Lonial[19] · Graham H. Jackson[20] · Kenneth C. Anderson[4] · Herve Avet-Loiseau[21,22] · Nikhil Munshi[4] · Anjan Thakurta[5] · Gareth Morgan[1]

FISH

+

Secuenciación

Double-Hit



	Events / N	18-Month Estimate
Node 8:ISS I, No Bi-allelic TP53 Nor Amp CKS1B	46 / 248	89% (84, 93)
Node 18:ISS II, No Bi-allelic TP53 Nor Amp CKS1B, Age < 65	29 / 139	81% (74, 88)
Node 11:ISS I/II, Wild type TP53	13 / 32	75% (59, 91)
Node 19:ISS II, No Bi-allelic TP53 Nor Amp CKS1B, Age >= 65	47 / 130	71% (63, 80)
Node 6:ISS III, No Bi-allelic TP53 Nor Amp CKS1B	87 / 187	65% (58, 73)
Node 10:ISS I/II, Bi-allelic or Mono-allelic TP53	12 / 21	46% (23, 69)
Node 7:ISS III, Bi-allelic TP53 and/or Amp CKS1B	21 / 27	33% (15, 52)

Log-rank p-value < .0001

✓Inactivación bi-alélica de TP53

✓ Amplificación de CKS1B (1q) (≥4 copias) en ISS III.

CONCLUSIONES

- ✓ El cell sorting aplica un potencial significativo en el campo clínico.
- ✓ La disponibilidad del cell sorting permite estudios accesorios
  (FISH, mutaciones puntuales, seuencias por Sanger, NGS, etc).
- ✓ Estas técnicas requieren determinada **pureza** y un número de **células mínimo** para su evaluación.
- ✓ Actualmente las entidades clinicobiológicas se definen con alteraciones moleculares indispensables para su definiciónen pacientes con patologías oncohematológicas.

✓La separación celular es indispensable en muestras con poblaciones neoplásicas escasamente representadas.

Gracias!!